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TECHNICAL NOTE

Highly polymorphic microsatellite loci for the Parsley frog (*Pelodytes punctatus*): characterization and testing for cross-species amplification

Mirjam S. van de Vliet · Onno E. Diekmann · Ester T. A. Serrão · Pedro Beja

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Abstract A microsatellite library was developed using genomic DNA of the Parsley frog, *Pelodytes punctatus*, an amphibian species which inhabits Mediterranean temporary pond systems. Number of alleles and heterozygosity ranged from 10 to 25 and from 0.66 to 0.90, respectively. Cross-species amplification was successful for 13 of the 15 developed loci for the related species, *Pelodytes ibericus*. The high levels of polymorphism revealed by these loci will be extremely useful for characterizing the population genetic diversity and structure and to estimate levels of dispersal and gene flow in the species *P. punctatus* and *P. ibericus*.

Keywords *Pelodytes punctatus* · Parsley frog · Microsatellites · Cross-species amplification · Temporary ponds · Amphibian conservation genetics

An excellent model to study metapopulation structures and their implications for conservation are amphibians inhabiting systems of temporary ponds in Mediterranean farmlands. Temporary ponds are small and shallow water elements with an annual dry phase of variable timing and duration, holding unique communities adapted to the limited duration of the wet period and absence of predators (Collinson et al. 1995). Mediterranean temporary ponds are suffering widespread degradation and loss due to increase in intensive cultivation and urban use. The destruction or direct modification of Mediterranean temporary ponds reduces dispersal possibilities and can cause reduction in population sizes and loss of genetic diversity. Restricted migration and consequent lower intrapopulation genetic diversity, has been associated to lower fitness in amphibians (Bridges and Semlitsch 2001; Rowe and Beebee 2002; Johansson et al. 2007). The Parsley frog (*Pelodytes punctatus*) is one of the adequate model species for investigating population structure in temporary ponds under different land use regimes. This species only breeds in temporary ponds with a hydroperiod of a few months, probably to avoid inter-specific competition and invertebrate predation (Beja and Alcazar 2003). We have developed 15 highly variable microsatellite loci, which can be used for measuring local migration, assigning individuals to their most likely population of origin and estimating effective population size.

Three genomic DNA libraries, enriched for different repeat length motifs were chosen to maximize the chance of obtaining loci with different polymorphism levels. Di- (CA, GA), tri- (CAC, CAG) and tetranucleotide (ATAG) repeat libraries were constructed using a slight modification of the procedures described by Gautschi et al. (2000), Garner et al. (2000). Genomic DNA from two tadpoles and one toe clip from an adult frog, collected from the south-west coast of the Algarve (Portugal), was isolated by using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). DNA was digested with *TSP*509I and fragments of 300–1,000 bp were isolated and ligated to TSPADSHORT/TSPADLONG linker sequences (Tenzer et al. 1999). DNA-linker fragments were amplified using TSPADSHORT as the PCR primer. PCR products were hybridized to 5′-

M. S. van de Vliet (✉) · O. E. Diekmann · E. T. A. Serrão
CCMAR, F.C.M.A—University of Algarve, Gambelas, 8005-139 Faro, Portugal
e-mail: mvliet@ualg.pt

P. Beja
ERENA, Ordenamento e Gestão de Recursos Naturais Lda, Rua Robalo Gouveia, 1-1A, 1900-392 Lisbon, Portugal

P. Beja
CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-66 Vairao, Portugal

biotinylated (Biotin-16-ddUTP, Roche) probe or a pool of two probes: $(CA)_{15}/(GA)_{15}$, or $(CAC)_{10}/(CAG)_{10}$ or $(ATAG)_8$ probes attached to streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, DYNAL biotech, Hamburg) at 65, 65 and 63°C, respectively. Enriched fragments were again amplified and products were ligated into pGEM-T Easy Vector (Promega), and transformed into *Escherichia coli* competent cells. Positive clones were amplified by polymerase chain reaction using universal primers T7 and SP6. PCR products were dot-blotted onto Nylon-Membranes (Hybond^{TM}-N^{+}, Amersham Pharmacia) and subsequently screened for microsatellites by oligonucleotide probes which were labelled with ECL3′-oligolabelling and detection system (Amersham Pharmacia).

Approximately 30% of the clones (161 positives of 536 totally screened) gave a strong positive hybridization signal. A total of 58, 23 and 80 inserts from di-, tri- and tetranucleotide libraries, respectively, were sequenced in an ABI prism 3130XL capillary sequencer. All inserts sequenced contained repeats, but $(ATAG)_n$ microsatellite isolation was most successful; 66% of the sequenced positives contained an $(ATAG)_{>10}$ repeat. For dinucleotide repeats (repeat number >10), isolation success was about 41% and surprisingly no long trinucleotide repeat motifs were found.

Primers were designed for inserts containing microsatellites with more than 12 single repeat type units (preferably with one unique repeat type in the fragment) and with flanking regions sufficiently large to allow primer development. A total of 23 primer pairs (7 and 16 for dinucleotide and tetranucleotide loci, respectively) were designed using mPRIMER3 software (http://bioinfo.ebc.ee/mprimer3/). Of these, seven were discarded for being monomorphic, for giving a wrong amplification product or no amplification product or for giving ambiguous multiple bands. For these seven loci amplification success could not be improved, even after using more stringent PCR conditions or redesigning primer pairs.

The 17 remaining primer pairs were used to genotype a set of 29 randomly chosen individuals from different separated populations in the Algarve and Alentejo, Portugal.

PCR amplifications were performed in a 10 µl reaction volume containing approximately 20 ng DNA, 1 µM primer and 0.1 µM of fluorescently labelled reverse primer, 2.0 mM $MgCl_2$, 5× Go*Tag* Flexi buffer (Promega), 0.2 mM of each dNTP and 0.75 U Go*Tag* DNA Polymerase (Promega). For 12 loci we used the following PCR program: 30–35 cycles with 95°C for 40 s, locus specific annealing temperature (Table 1) for 40 s, and 72°C for 40 s. The touchdown program for the other three loci PCR amplification held two cycles of 30 s at 94°, 30 s at 62°C, 30 s at 72°C, followed by 14 cycles of 0.5°C touchdown: 15 s at 94°C, 15 s at 62–55°C, 15 s at 72°C, followed by 24 cycles of 15 s at 94°C, 15 s at 55°C, 15 s at 72°C. For all PCR reactions we started with a denaturation step of 95°C for 5 min and the last cycle was followed by a 7 min extension. Fragment size was determined on an ABI prism 3130XL capillary sequencer. Allelic diversity, observed (H_O) and expected heterozygosities (H_E), deviation from Hardy–Weinberg equilibrium and linkage disequilibrium were estimated using GENETIX ver. 4.04 (Belkhir et al. 2000).

Two of the 17 loci were subsequently discarded because they regularly gave more than two alleles per individual. The 15 remaining microsatellites produced clear consistent amplification products and were highly polymorphic, showing also high allelic diversity between individuals within a population. Table 1 summarizes the characteristics of these primer pairs. In order to prevent misinterpretation of allele banding pattern we selected loci which only give clear and consistent allele banding patterns with little 'stutter' in order to reduce any possible confusion between homozygote and adjacent allele heterozygote genotypes. For all tetranucleotide microsatellite loci extra 'stutter' bands were always low, which makes genotyping easier and more reliable compared with the dinucleotide repeat loci (*Ppu*1 and *Ppu*2 have a 'stutter' pattern; *Ppu*15 has not). Genotyping errors provide individuals with an incorrect multilocus genotype and can cause substantial errors, especially in parentage studies or assigning individuals to the right population (Hoffman and Amos 2005). Number of alleles ranged from 10 to 25 and observed heterozygosities ranged from 0.66 to 0.90 and did not deviate significantly from expected values. No linkage disequilibrium was detected among any pair of loci. There was no evidence for allelic dropout or for null alleles using software MICRO-CHECKER (Van Oosterhout et al. 2003).

Because the developed microsatellite markers might also be useful for population genetic studies in related species, we tested them with seven individuals from different populations of *Pelodytes ibericus*. Cross-species amplification was also tested with another member of the same Superfamily (Pelobatoidea): *Pelobates cultripes* (four individuals) and two other more distant related amphibian species *Hyla meridionalis* (four individuals) and *Pleurodeles waltl* (four individuals). We used the same PCR conditions as performed for *P. punctatus*. When amplifying DNA from the related species *P. ibericus* all loci except for *Ppu*12 and *Ppu*13 produced clear amplification products near the expected size range and are highly variable (Table 2). This result is rather promising for population genetic studies in *P. ibericus*. Amplification failure in the other three species confirmed poor cross-species amplification success in amphibians (Primmer and Merilä 2002; Rowe et al. 1997, 2000; Garner 2002 Garner et al. 2003).

The high numbers of alleles per locus indicate the potential usefulness of these loci to characterize the

Table 1 Fifteen polymorphic microsatellite loci for the species *Pelodytes punctatus*

Locus	Primer sequence (5′-3′)	Repeat structure	No. of alleles	Size range (bp)	T_a (°C)	H_E	H_O
*Ppu*1	AAAATCTTATAGTTCAAACCTCCTAACAA CCTCTTATCTCCCAATCACTTCC	$(AC)_{31}$	20	117–177	52	0.93	0.83
*Ppu*2	CCAGAAAGTCGCTGTTTCAGAGCAGG GCCCAGCATGGATATTGGTTTTGTGTG	$(CA)_{17}(CCCTCA)_4$	16	212–332	60	0.86	0.89
*Ppu*3	TGTTTCTGGTTGTCTCCTTGCAGCTTTG AGTCAGCATCATAGGAAATATGGGCTCTTTGG	$(ATCT)_{18}$	16	308–356	55	0.87	0.79
*Ppu*4	AACACTCTTATGGGCTCCTGGTTT CTGCTCCGTTGTGCTAATGGG	$(ATCT)_{17}ATCC(ATCT)_3AACAT(AC)_3$	12	252–298	52	0.87	0.79
*Ppu*5	CGTCAAGATGTTAAAGGTATAGGCAGGT AGACAGATACAGAACGATTGGGAAAGG	$(TCTA)_{13}(TCTG)_{18}(TCTA)_6TC(TCTA)_2$	24	236–412	55	0.92	0.82
*Ppu*6	TGGCAAAGATAACAGACTTGGCGTG AGGCTTGAAGACCATAAAAAGAGAGGACCA	$(TATC)_{18}$	11	89–133	60	0.89	0.74
*Ppu*7	GTACATCAGAGATATGGCGCTTTCGTT TCCGCTACTAGAACCTATCCATCCCT	$(AGAT)_{18}$	12	178–220	55	0.89	0.82
*Ppu*8	ATGGCCATGTATACACAAATGTATTTAACAAA TCAAGGCAAGGTACAAGAGACAACA	$(CTAT)_4(CTTT)_2(CTAT)_{25}$	25	168–306	Tdown	0.94	0.87
*Ppu*9	GGCTTACTATCAATAATCATCTGTGCATGTT TGTAAGTAGCTATTGCTGTTTGCAGT	$(ATCT)_{14}$	11	192–250	55	0.87	0.72
*Ppu*10	GTGCACAGTGTGTCCTAAATGT GGTACAATGAGCAAGGTTCCAG	$(ATCT)_{14}$	15	96–160	Tdown	0.87	0.79
*Ppu*11	TTCAAGTGTAGGAGCACCGAGAGG AGAAGACACACAGATAGACAGAAGCAGAG	$(TCTA)_{14}$	11	137–189	Tdown	0.83	0.72
*Ppu*12	TGTCATATAGTCTCTTTTGTTCTGCAGTT CAGCCTGGGGTACTGTATTATTCTATGT	$(ATAG)_4ATGG(ATAG)_{12}$	15	264–328	52	0.89	0.79
*Ppu*13	ACCTCTGTGCTCTACCCTTCCTCC AGAGGAGTAAGCCAAGGGATATGTATAGGA	$(TG)_4(TATC)_{13}$	11	114–142	55	0.86	0.81
*Ppu*14	ACAAATACACCCAGGAGAAGGGAGACA ATGGGGCTTGGTCGGGGTAAGAG	$(TAGA)AAGA(TAGA)_{16}$	12	161–205	60	0.87	0.90
*Ppu*15	AGTAAATAACATAATCCCTGCCCTTGC AGCTACAAAGAGAAGCAAAACATGGC	$(TG)_{15}$	10	101–121	55	0.76	0.66

Allelic data and genetic variation were derived from 29 individuals of *Pelodytes punctatus* from different separated locations along the south-west coast of Portugal. T_a, locus-specific annealing temperature; H_O, observed heterozygosity; H_E expected heterozygosity. Size range refers to the PCR product size at each locus. The characteristics of the repeat are based on the sequenced clones (GenBank Accession numbers EU667612-EU667626)

Table 2 Cross-species amplification results for seven individuals of *Pelodytes ibericus*

	Amplification success	Size range (bp)	No. of alleles
Ppu1	++	115–163	1
Ppu2	++	218–284	6
Ppu3	+	332–428	9
Ppu4	++	240–274	7
Ppu5	++	304–476	10
Ppu6	++	105–137	6
Ppu7	++	178–228	8
Ppu8	++	238–266	6
Ppu9	++	192–212	5
Ppu10	++	86–124	7
Ppu11	++	157–193	6
Ppu12	−		
Ppu13	−		
Ppu14	++	177–197	6
Ppu15	++	99–101	2

++, Indicates very good amplification; +, indicates good amplification but needs further optimization; −, indicates ambiguous amplification and multiple bands or no amplification. Size range refers to the PCR product size at each locus. Amplification conditions are similar to *P. punctatus*

population genetic structure and dispersal behaviour of *P. punctatus.* These loci presented here therefore provide a valuable tool for the conservation genetics analyses of populations in temporary ponds under human pressure.

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